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# Host and Environmental Factors Influencing Individual Human Cytokine Responses

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## SUMMARY

Differences in susceptibility to immune-mediated diseases are determined by variability in immune responses. In three studies within the Human Functional Genomics Project, we assessed the effect of environmental and non-genetic host factors of the genetic make-up of the host and of the intestinal microbiome on the cytokine responses in humans. We analyzed the association of these factors with circulating mediators and with six cytokines after stimulation with 19 bacterial, fungal, viral, and non-microbial metabolic stimuli in 534 healthy subjects. In this first study, we show a strong impact of non-genetic host factors (e.g., age and gender) on cytokine production and circulating mediators. Additionally, annual seasonality is found to be an important environmental factor influencing cytokine production. Alpha-1-antitrypsin concentrations partially mediate the seasonality of cytokine responses, whereas the effect of vitamin D levels is limited. The complete dataset has been made publicly available as a comprehensive resource for future studies.

## INTRODUCTION

Host defense mechanisms mediated by the immune system protect the host from the invading pathogens that continuously attempt to breach the mucosal and skin barriers. Individuals with diminished immune responses from inborn or acquired causes have an increased susceptibility to infections (Blot et al., 2002; Clark and Hajjeh, 2002; Fishman, 2007; Fishman and Rubin, 1998; McNeil et al., 2001). Conversely, individuals with an overactive immune system are more susceptible to autoimmune diseases such as rheumatoid arthritis, type 1 diabetes and multiple sclerosis, and inflammatory diseases such as gout, Crohn's disease, and atherosclerosis (Gandhi et al., 2010; Martinon, 2010; Szablewski, 2014). Variability in the immune responses also influences susceptibility to other important pathologies such as malignant processes (de Visser et al., 2006) and neurodegenerative diseases like Parkinson's disease and Alzheimer's disease (Heneka et al., 2015; Mosley et al., 2012). This variability in immune responses is likely affected by factors already known to influence disease prevalence such as age, gender, and seasonality. For instance, women are more likely to suffer from autoimmune diseases, it gets more difficult to fight off infections with age, and influenza infections peak in winter. However, the differences in susceptibility to immune-mediated diseases between individuals cannot be fully accounted for by what is currently known, and a systems biology-based approach

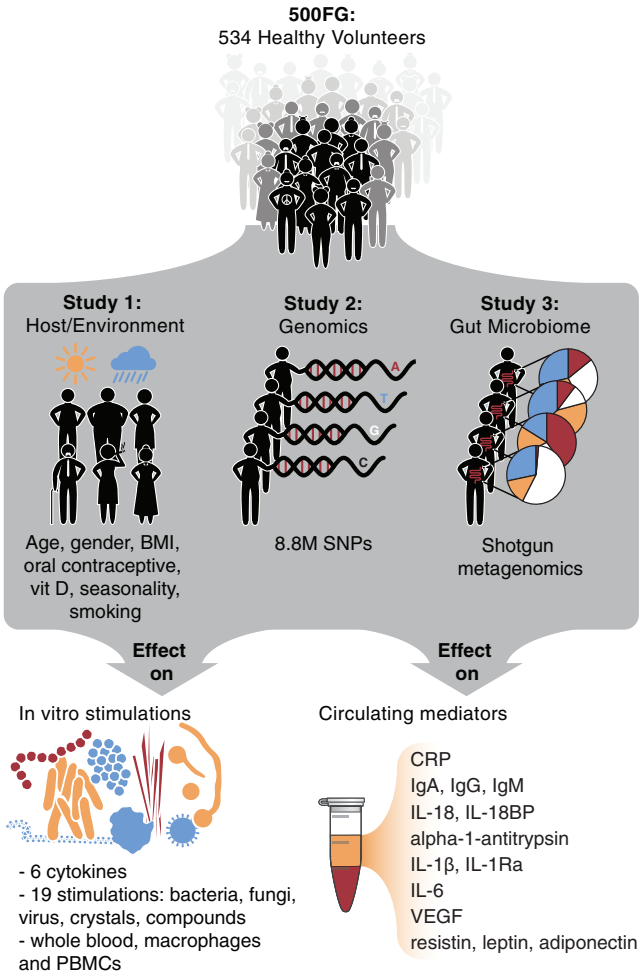


**Figure 1. Schematic Overview of the Three Studies on the 500FG Cohort**

The cohort consists of 534 volunteers with varying characteristics. The current manuscript is the first in a series of three studies presented in this issue of *Cell*, that aim to provide a systematic assessment of the impact of various intrinsic and environmental factors (this manuscript), the host genome (Li et al., 2016), and the gut microbiome (Schirmer et al., 2016) on cytokine production and baseline immune parameters in a systems biology-based approach.
See also Figure S1 and Tables S1 and S2.

to comprehensively assess the environmental and host-related factors that influence immune responses is needed.

Production and release of proinflammatory cytokines is one of the most important components of host defense mechanisms, representing the communication network within the immune system. So far, variation of cytokine production capacity in the general population has been investigated only in small studies, and this limitation resulted in conflicting conclusions (Aulock et al., 2006; Bernstein and Murasko, 1998; Grandgirard et al., 2013; Hwang et al., 2015; Nielsen et al., 2013; Scott et al., 2013). The only large-scale studies of the immune system published to date are genome-wide studies focused on the regulatory effect of genetic variation on cytokine gene transcription levels (eQTLs) rather than on protein expression levels (Berry et al., 2010; Fairfax et al., 2014; Lee et al., 2014; Raj et al., 2014; Ye et al., 2014). These studies were based on a limited number of stimulations or used unstimulated cells. The few studies assessing variability of cytokine production are all based on responses to standard immune stimuli such as lipopolysaccharide (LPS) (Fairfax et al., 2014; Lee et al., 2014). A comprehensive assessment of environmental and host factors influencing cytokine responses to a wide range of pathological and physiological stimuli is still lacking.

The Human Functional Genomics Project (HFGP; www.humanfunctionalgenomics.org) aims to fill this gap. Within HFGP, the first major study that intends to assess the variability of human cytokine responses is the 500 Functional Genomics (500FG) study, comprising of a group of ~500 healthy volunteers of Western-European ancestry. The specific aim of the 500FG cohort is to assess and integrate the various factors influencing individual human cytokine responses. The 500FG study has two important advantages compared to previous studies: first, it comprises the largest cohort of healthy individuals studied to date, and second, the cytokine production was assessed in response to a large panel of microbial and metabolic stimuli and in three different cellular systems. The current manuscript is the first in a series of three studies presented in this issue of *Cell*, that aim to provide a systematic assessment of the impact of various intrinsic and environmental factors (this manuscript), the host genome (Li et al., 2016), and the gut microbiome (Schirmer et al., 2016), on baseline immune status and cytokine production in a systems biology-based approach. A schematic overview of these three studies on the 500FG cohort is presented in Figure 1.

In this first study, we focus on environmental and non-genetic host factors that have been described to influence the immune response and/or disease prevalence (e.g., age, gender, BMI, oral contraceptive usage, smoking, vitamin D, and seasonality), but for which a comprehensive assessment of their effect on cytokine responses is missing. We observe that several of the studied factors directly influence immune parameters and host defense and we describe a novel vitamin D-independent/alpha-1-antitrypsin (AAT)-dependent effect of seasonality on inflammation.

## RESULTS

### Measurement of Circulating Immune Parameters

To study variation in baseline immune status, we measured circulating concentrations of some of the most important families of parameters responsible for host defense in the circulation: acute phase proteins (CRP and AAT), immunoglobulins (IgA, IgM, IgG, and 4 $IgG^+$ sub-classes), adipokines (leptin, adiponectin, and resistin), IL-6, IL-18, IL-18-binding protein (IL-18BP), IL-1β, and interleukin-1 receptor antagonist (IL-1Ra). Measurement of resting levels of low abundance cytokines such as IL-1β, IL-6, IL-18, and VEGF are below the lower limit of quantification by standard ELISAs in a healthy cohort; therefore, the Ella microfluidic analyzer was used to assess cytokine concentrations in the fg/mL to low pg/mL range. For instance, the mean level of IL-6 concentrations of the cohort was 1.25 ± 0.06 pg/mL (range 0.15–8.1 pg/mL; see the STAR Methods for levels of all circulating mediators). The assessment of these parameters provides

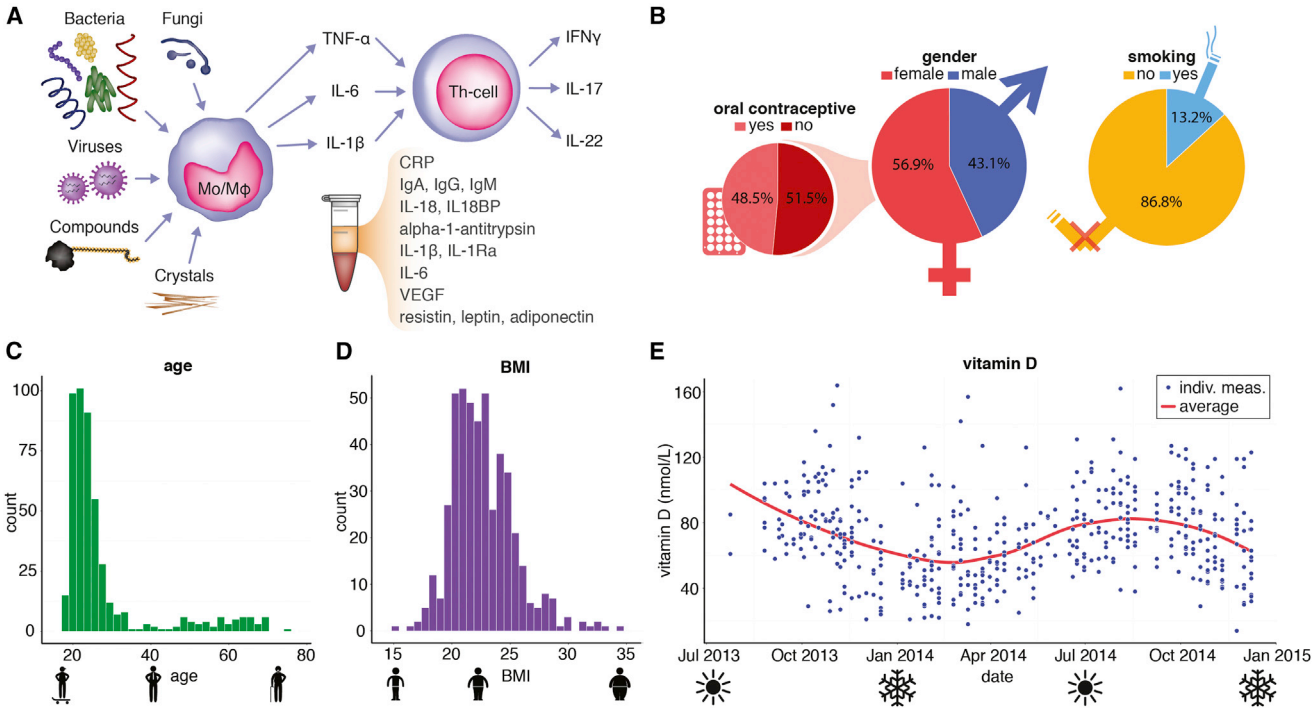


**Figure 2. Schematic Depiction of the Study Parameters**

(A) Stimuli and measurements in this study.

(B) Pie charts showing some characteristics of our cohort; from left to right, the percentage of women using oral contraceptives, the percentage of men and women, and the percentage of people that are active smokers.

(C) Histogram showing the age distribution in our cohort.

(D) Histogram showing the BMI distribution in our cohort.

(E) Scatterplot of the vitamin D levels for all individuals plotted against the date which they provided blood samples. The blue dots are the individual vitamin D measurements and the red line is the LOESS fit through these points.

See also Figure S1 and Tables S1 and S2.

a comprehensive view of baseline immune characteristics, most importantly of inflammatory status and humoral immunity.

The immune parameters were analyzed as a function of a set of environmental and intrinsic non-genetic host factors (age, gender, BMI, oral contraceptive usage, smoking, vitamin D concentrations, and seasonality) (Figure 2).

## Correlations between Circulating Cytokine Concentrations Indicate Co-regulation

As displayed in Figures 3A, 3B, and S2A, some cytokine levels show positive correlations with other cytokine levels. IL-1β concentrations show a strong correlation with IL-6 ($p < 0.0001$), a particularly relevant observation, because treatment of patients with IL-1β blocking therapies reveals a consistent fall in circulating IL-6 levels (Dinarello et al., 2012). IL-1Ra and IL-6 concentrations also correlate significantly because these are both markers of inflammation (Figure 3B). IL-1β circulating concentrations also correlated with IL-1Ra ($p < 0.05$), and this is in agreement with in vitro and human trial data demonstrating that IL-1β induces its own receptor antagonist. In line with the well-known anti-inflammatory effects of AAT (Bergin et al., 2012; Lewis, 2012), the only strong negative correlation was found between AAT and IL-1β concentrations (Figure 3B).

## Age and Gender Have a Significant Impact on Circulating Mediators

The concentrations of IL-6 and IL-1Ra are increased in the circulation of older individuals (Figure 3C, with relevant examples in Figure 3D). An increased low-level inflammation during the aging process (inflammaging) has been proposed as a culprit for metabolic syndrome and cardiovascular diseases (Kovacic et al., 2011a, 2011b; Veronica and Esther, 2012), and in our study, the higher IL-6 and IL-1Ra concentrations with age gives weight to this hypothesis. The effect of age on IL-6 levels is also supported by the validation of these findings in a second independent cohort of 300 individuals from the HFGP ($p = 1.12e\text{-}03$, Figure S2E). With regard to gender, we find significantly higher circulating concentrations of IL-1Ra in women. This was previously observed in a cohort of type II diabetes patients (Ybarra et al., 2008), which we now confirm in healthy individuals.

We also observe an increase in IL-18BP concentrations in older individuals (Figure 3C), and IL-18BP levels were also significantly higher in men than in women, while total IL-18 concentrations remained unaffected by both age and gender. However, in patients with cardiovascular disease, IL-18 levels are higher in men than in women (Opstad et al., 2011).

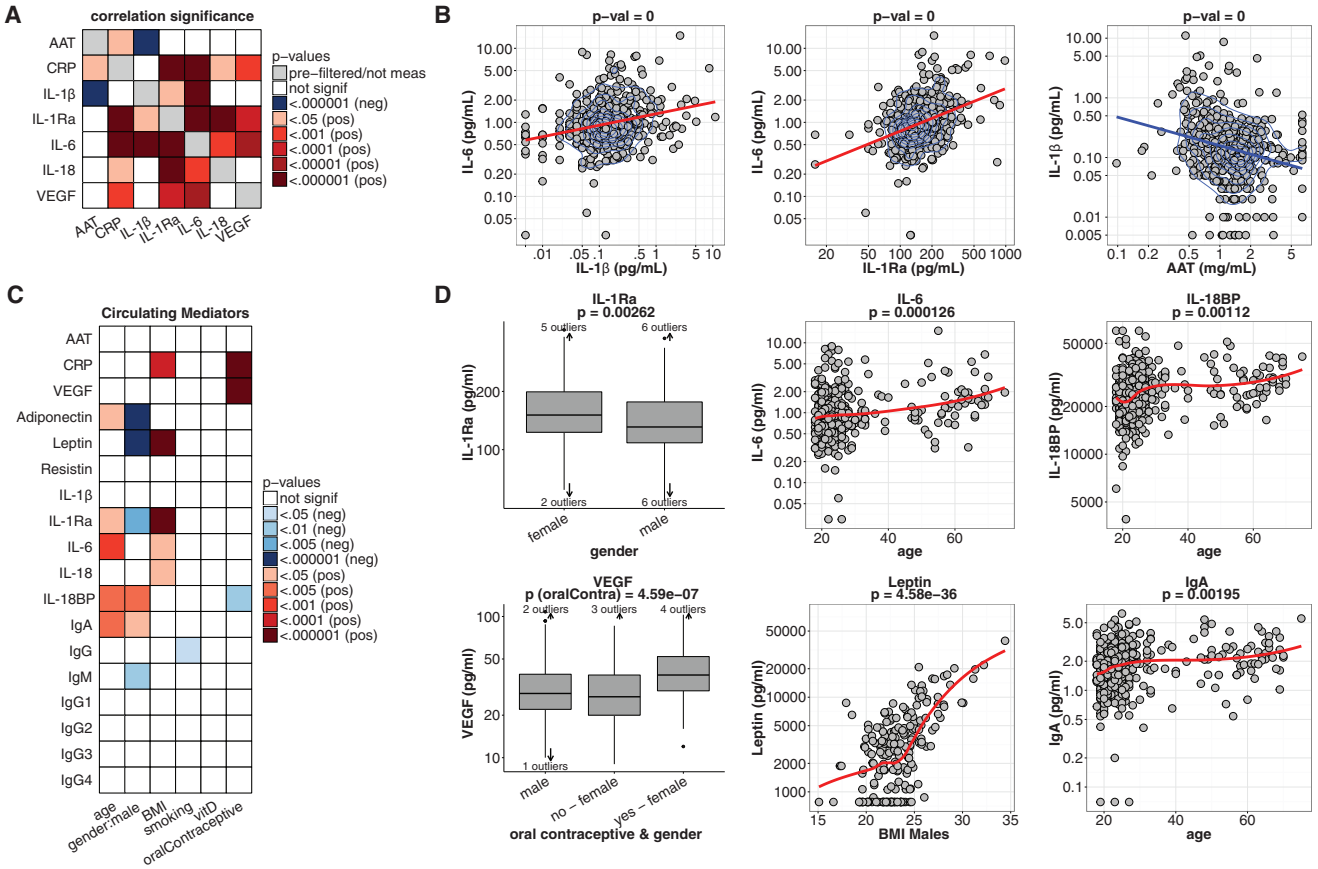


**Figure 3. Correlations of Circulating Mediators with Non-genetic Host Characteristics**

(A) The p values (FDR corrected) of the correlations of mediators with each other. For color codes of the FDR, see legend.

(B) Scatterplots of highly significant correlations from (A). The thin blue lines are contour lines, indicating the density of the scatterplot.

(C) Significance (FDR corrected) of the relation between host characteristics/environmental factors (x axis) to circulating mediators and immunoglobulins (y axis).

(D) Scatterplots showing examples of the effect of age, gender, oral contraceptive and BMI. The lines indicate the local regression (LOESS) fit.

See also Figure S2 and Table S3.

IgA immunoglobulin concentrations also increase with age (Figure 3C), as was previously detected in saliva (Gonzalez-Quintela et al., 2008; Jafarzadeh et al., 2010). IgA has been implicated with several age-related diseases such as macular degeneration and diabetes, which makes IgA a potential therapeutic agent for prophylaxis and/or treatment (Rodriguez-Segade et al., 1996; Yu et al., 2016). Higher IgA was also detected in men compared to women, as supported by earlier studies, while circulating concentrations of IgM were higher in women (Cassidy et al., 1974; Obiandu et al., 2013; Weber-Mzell et al., 2004). In another study from the HFGP (Aguirre-Gamboa et al., 2016), IgG2 and IgG4 are shown to significantly interact with age and gender, respectively. Due to the larger number of factors corrected for, and stronger multiple testing correction applied in this manuscript, we observe only a borderline significant effect. However, using similar corrections we find false discovery rates (FDRs) of 4.06e-3 and 2.20e-3 for IgG2 and IgG4, respectively.

Concentrations of leptin and adiponectin are higher in women than in men (Figure 3C). This is due to the higher percentage of adipose tissue in females compared to males, as well as a higher secretion rate of leptin in females compared to males as previously shown by others (Böttner et al., 2004; Considine et al., 1996; Hellström et al., 2000). Adiponectin levels were also raised in older individuals. CRP levels show a positive association with the use of oral contraceptives, as shown by several earlier studies (Buchbinder et al., 2008; Cauci et al., 2008; van Rooijen et al., 2006). In addition, plasma VEGF was higher in women using oral contraceptives (Charnock-Jones et al., 2000; Macpherson et al., 1999) (Figure 3C), whereas circulating IL-18BP were lower. These findings provide clinical validation that biomarkers such as circulating low abundance cytokines reflect fundamental physiologic parameters in the absence of disease.

## Stimulation of Proinflammatory Cytokines in Three Ex Vivo Systems

To comprehensively capture cytokine responses of immune cells, we measured the production of both monocyte-derived (IL-1β, TNF-α, IL-6) and lymphocyte-derived cytokines (IFNγ, IL-17, IL-22) after stimulation in three ex vivo systems (whole blood, peripheral blood mononuclear cells [PBMCs], and

monocyte-derived macrophages) with 1 of 19 stimuli (eight bacterial, four fungal, one virus, four purified microbial ligands, and two metabolic stimuli). We used these complex cellular systems with 24-hr (monocyte-derived cytokines) and 7-day (lymphocyte-derived cytokines) stimulation times in order to mimic real-life situations, as opposed to artificial systems using purified cell populations. This resulted in a total of 128 cytokine measurements for each of the 534 individuals included in this study. This is by far the largest set of stimuli-cytokine combinations measured to date, with previous studies generally studying one or two stimuli in one experimental setting. In addition, the choice was made to focus on protein levels (that confer biological activity) rather than gene expression (as in earlier eQTL studies), because protein levels are a better representation of an individual's immune state. Gene expression and protein expression levels of cytokines are not always correlated to one another, because post-transcriptional processes play a crucial role in determining the rate of cytokine synthesis and release (Anderson, 2008). A complete list of measurements is provided in Table S1, and example distributions are shown in Figure S1. The data for each participant (including all measurements of circulating immune parameters) can be found at https://hfgp.bbmri.nl/ (database is described in detail in the STAR Methods).

### IFNγ and IL-22 Responses Decrease with Age

Production of monocyte-derived cytokines (IL-1β, TNF-α, and IL-6) after stimulation was similar across the ages of the volunteers, with the exception of *Staphylococcus aureus*-induced IL-1β and *Candida albicans* hyphae-induced IL-6, that showed a moderately higher production in the elderly. In contrast, there was a consistent effect of age on lymphocyte function, with the production of IFNγ and IL-22 being significantly lower in elderly individuals after stimulation with most pathogens, in line with the concept of immune-senescence (Baylis et al., 2013). Interestingly, no such impairment with age was observed for the production of IL-17, an important product of Th17 cells. The strongest decrease of cytokine production in the older participants was observed after stimulation with *Borrelia spp*., as detailed and analyzed in the HFGP manuscript by Oosting et al. (2016). The impact of age on cytokine production capacity induced by various microbial and metabolic stimuli is schematically depicted in Figure 4A, with relevant examples provided in Figure 4C.

### Monocyte-Derived Cytokine Responses Are Increased in Men, whereas Women Have Increased Th17 Responses to *Candida*

The production of proinflammatory cytokines released from monocytes was higher in men after stimulation with several stimuli. In the whole-blood system, these cytokines were increased in men after LPS stimulation, while in PBMCs this effect was apparent especially after stimulation of *C. albicans* conidia. Figure 4B presents an overview of the gender effects on cytokine production, with examples shown in Figure 4E. Although the use of oral contraceptives did not have strong effects on cytokine production capacity in vitro, women using oral contraceptives did show an even further decreased IFNγ and TNF-α response after LPS stimulation (Figures 4D and 4E). Th17 responses were mostly similar between men and women, although IL-17 and IL-22 production was higher in women after stimulation with *C. albicans* hyphae (Figure 4B).

### Hormonal Differences Do Not Explain Gender-Specific Immune Differences

To investigate whether differences in circulating hormone levels may have a role in the above-described gender effects, we assessed the correlation of inflammatory markers with the levels of progesterone and testosterone in men and women separately. The majority of the cytokines and mediators identified to differ between men and women showed no correlation with progesterone and testosterone concentrations, excluding a potential role of hormones in explaining the gender differences. However, we identified several notable exceptions, with the significant correlations displayed in Figure 4F (all correlations in Figure S2C). One of the most important findings is that leptin concentrations show a clear negative correlation with testosterone levels within the male subgroup of the 500FG cohort ($R = -0.36$, FDR = 6.78e-06, see Figure 4G), supported by previous reports (Behre et al., 1997). Surprisingly, however, no such effect is observed in women. In fact, if anything, there is a positive correlation between leptin levels and testosterone (FDR = 0.07; Figure S2D), in line with a study that observed a similar effect for non-obese women (Soderberg et al., 2001). Moreover, there was a significant positive correlation and a significant negative correlation of testosterone and progesterone levels, respectively, with circulating IL-18BP concentrations in men, which was partially able to explain the gender difference.

### Smoking and BMI Do Not Affect In Vitro Cytokine Production, but BMI Influences Several Circulating Mediators

We have analyzed two sets of parameters in relation with non-genetic host factors: resting circulating concentrations of the immune mediators and in-vitro cytokine production capacity after microbial stimulation. Surprisingly, with the exception of a few spurious effects, BMI and smoking had no detectable effect on in vitro cytokine production (Figures S3A and S3B), even though they are thought to be important modulators of immune responses (McCrea et al., 1994; Sopori, 2002). In contrast, some of the circulating mediators measured were significantly related to BMI (Figure 3C). As expected, leptin and CRP correlated positively with BMI (Buchbinder et al., 2008; Cauci et al., 2008; van Rooijen et al., 2006). BMI also showed a small but significant association with circulating levels of both IL-6 and IL-18, which is in accordance with the concept of an increased inflammatory status when BMI increases (Kantor et al., 2013; Khaodhiar et al., 2004; Siervo et al., 2012). This finding was confirmed in an independent cohort of volunteers ($p = 4.3e-5$, Figure S2A). The same increased inflammation is likely the cause of the increased IL-1Ra concentrations in individuals with high BMI: the levels of the cytokine are probably reactively upregulated. The only effect we observe for smoking is a reduction in IgG levels, which confirms the findings of a study by Gonzalez-Quintela et al. (2008).

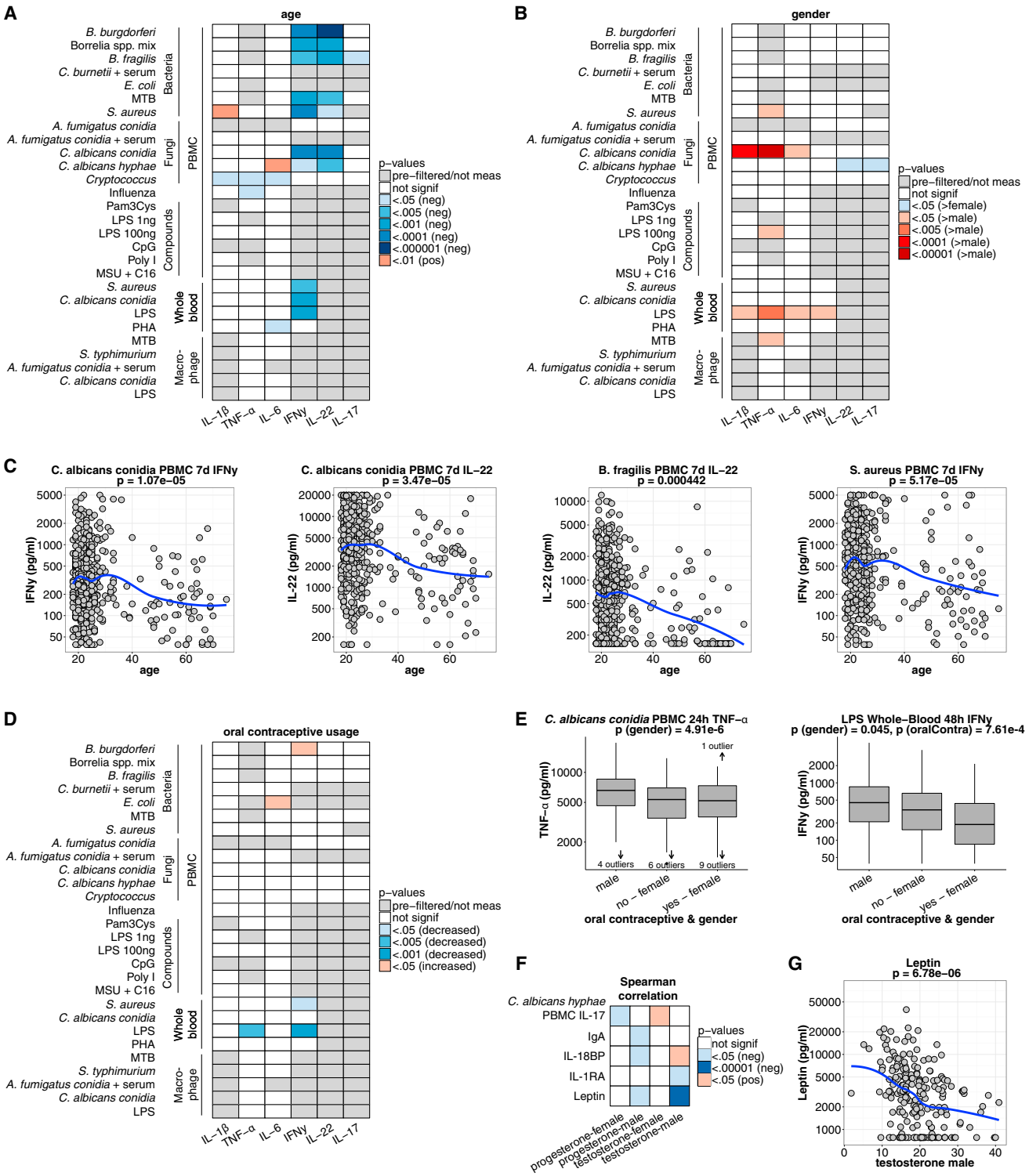


**Figure 4. Relation of Age, Gender, and Oral Contraceptive Usage to Cytokine Production**

(A) Significance of age in relation to different cytokines (x axis) induced by different stimuli (y axis), all values have been FDR-corrected. The darker the color, the greater the significance, where a decrease with age is blue and an increase is red (see figure legend).

(B) Similar plot to Figure 3A for gender. Red indicates a stronger response in men, whereas blue indicates a stronger response in women.

(C) Specific correlations of age to IFNγ and IL-22 production with different stimuli (the lines indicate the LOESS fit).

(D) Similar to Figure 4A and 3B, for the effect of "oral contraceptive usage," where red indicates an increase with usage of oral contraceptives and blue a decrease.

*(legend continued on next page)*

### Seasonality Has a Major Impact on Cytokine Responses and Inflammation

It was recently suggested that gene expression in human immune cells shows annual seasonality (De Jong et al., 2014; Dopico et al., 2015). We therefore checked immune responses for the presence of annual seasonal patterns using linear regression. This analysis provided both the significance of the seasonality pattern and the month at which the immune responses were at their highest (Table S3). Results were confirmed using an independent nonlinear fitting method (Table S4). The results show that the production of several cytokines (TNF-$\alpha$, IL-1$\beta$, and IL-6) shows a significant peak in summer (Figure 5A), while circulating AAT concentrations were highest in the winter (Figure 5C). The clearest effects of seasonality are apparent for monocyte-derived cytokines after stimulation with influenza virus, *Coxiella burnetti*, or *Cryptococcus neoformans* (Figure 5D). As influenza incidence displays a very clear seasonality pattern, with increased transmission in the winter months (Lipsitch and Viboud, 2009; Lofgren et al., 2007), it is tempting to speculate that the lower cytokine responses to influenza in the winter months may represent an important pathophysiological factor in this phenomenon.

The extent to which seasonal variation in baseline gene expression could contribute to seasonal variation in cytokine responses remains to be fully tested. To get an estimation of this contribution, we performed RNA-sequencing in a subset of 88 volunteers and first analyzed the seasonality patterns of AAT and the three cytokines showing seasonal responses (IL-1$\beta$, TNF-$\alpha$, IL-6; Figure 4B). Only TNF-$\alpha$ mRNA showed a seasonal pattern of expression, peaking in summer, which matches protein secretion (Table S5). Additionally, we checked the same genes in a large pediatric cohort from Germany, which shares a very similar climate with the Netherlands (Dopico et al., 2015). PBMC expression of *SERPINA1* (the gene encoding AAT) was found to be seasonal in that cohort. It is important to note that AAT seasonality is among the weaker seasonal genes in the German cohort of Dopico et al., (2015) in which 5,136 genes were found to be seasonal (corresponding to $\sim$1/4 of protein-coding genes in the human genome). Second, we assessed seasonality of mRNA expression of genes known to be involved in regulating cytokine production: PRRs, signaling molecules, and transcription factors (for details see the STAR Methods). Expression analysis of these genes in our cohort showed no individual seasonal patterns after multiple-testing correction (Table S6). Interestingly, in the genes significant before correction, there was an enrichment of seasonal genes peaking in summer (19 out of 30 genes, chi-square $p < 0.01$), the same season at which concentrations are highest for most seasonal cytokines. Inspection of this same regulatory set of genes in the larger German pediatric cohort revealed a larger set of seasonal genes involved in the regulation of cytokine expression (Table S7). Seasonality of these regulatory genes, combined with marginal seasonal gene expression of AAT and one out of three seasonal cytokines, does point toward some transcriptional regulation. However, this also suggests that post-transcriptional processes, rather than gene transcription, are influenced strongly by seasonal variations. Additionally, the biological relevance of RNA levels is less direct, and more difficult to interpret, than protein expression levels. This strengthens our choice to investigate cytokine expression as a more direct measure of immune regulation.

### Variations in Vitamin D Concentrations Have Limited Effect on Cytokine Production Capacity

Vitamin D has been repeatedly reported to have important immunomodulatory effects (Bikle, 2009; Correale et al., 2009), and based on this, we expected to observe important effects on cytokine production capacity. Surprisingly, vitamin D circulating concentrations did not have a significant effect on any of the cytokine production systems investigated here (Figure 5B). The seasonality of vitamin D has been suggested to influence inflammatory markers (Prietl et al., 2013): however, when we separated out the general periodicity over time and the residual variation of vitamin D at each time point, it was only the periodicity that was significantly correlated with cytokine outcomes and not the season-independent vitamin D variations (Figures S3C and S3D). This is not due to a lack of residual variation around the periodic signal, because the residuals have an amplitude similar to the periodic signal (Figure 2E). With inter- and intra-assay coefficients of variation of <5%, the residuals can also not be ascribed to measurement noise. There are a number of environmental factors that show seasonal variations, which explains why vitamin D concentrations alone are not a strong predictor of cytokine responses. Examples of factors that peaked in summer include temperature and atmospheric $NH_3$ and $O_2$ levels, whereas humidity and $SO_2$, NO, $NO_2$, and CO concentrations peaked in winter (Figure S4). Pollen, which is a known allergen, peaked in concentration at different times during our study depending on the species and could also have contributed to seasonal immune responses (examples in Figure S4). It is thus likely that vitamin D is only one of many other factors with a seasonal pattern that influence the immune response.

### Alpha-1-Antitrypsin Is Partially Responsible for Seasonality of Cytokine Responses: Impact on Gouty Inflammation

In contrast to in vitro cytokine production, most circulating markers were not influenced by the season, with the notable exception of plasma alpha-1-antitrypsin (AAT) concentrations, which were highest in February and lowest in the summer months (Figures 5C and 6A). Thus, the periodicity of AAT is opposite to the periodicity of the cytokines that are highest in summer (displayed in Figure 6B for IL-1$\beta$ after stimulation with MSU + C16 and Figure S5 for others). AAT is a known anti-inflammatory mediator (Bergin et al., 2012; Joosten et al., 2016), but to the best of

(E) Boxplot visualization of cytokine production with gender/oral contraceptive usage.
(F) Correlations between progesterone/testosterone levels and cytokine/IgA levels for men and women. Tests were performed for all immunological responses showing a significant relation to gender. Only the resulting significant correlations are shown in this plot.
(G) Scatterplot of the relation between leptin and testosterone in men, where the lines indicate the LOESS fit.
See also Figures S2 and S3 and Tables S1 and S3.

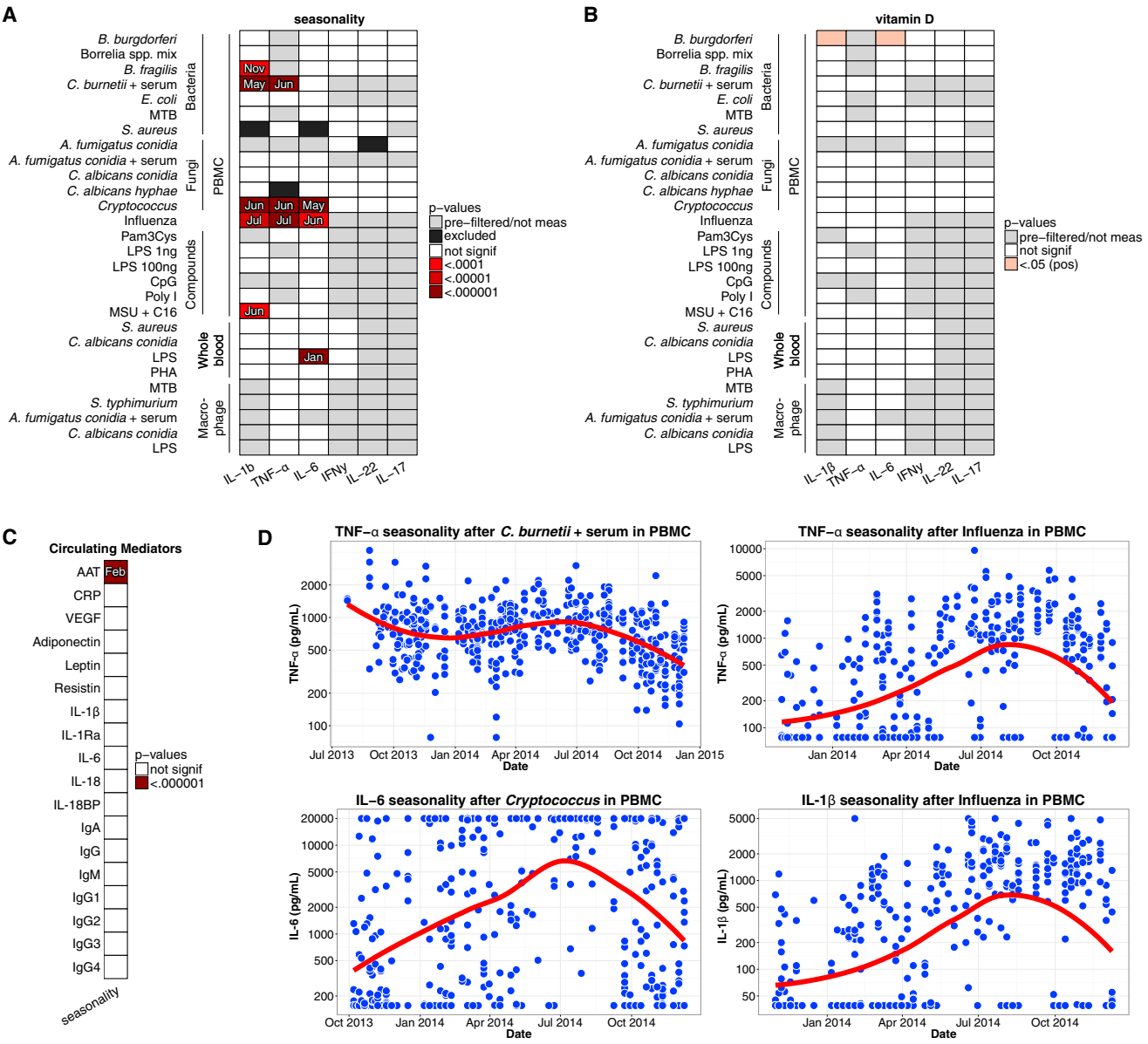


**Figure 5. Seasonal Changes in Cytokine Levels**

(A) Heatmap showing the cytokines having seasonal responses. Three letter abbreviation indicate the month at which the production of the cytokine (x axis) is highest. A few stimulus-cytokine combinations were excluded (see legend), because the time profile showed clear storage degradation effects which interfere with the seasonality analysis.

(B) Effects of vitamin D levels shown in a heatmap similar to Figure 3A, red indicates a positive relation.

(C) Heatmap showing the significance of the seasonality of the circulating cytokines.

(D) Examples of some of the seasonal responses, where each blue dot is an individual measurement and the red line depicts the LOESS curve.

See also Figures S2, S3, S4, and S5 and Tables S3, S4, S5, S6, and S7.

our knowledge, nothing is known about its seasonal variation. Because of the opposite seasonality of AAT with cytokine induction by uric acid crystals and fatty acids (C16) (Figure 6B), we validated the impact of AAT on cytokine response in sterile inflammation in an experimental model of gout, a condition caused by the deposition of MSU crystals in the joints. When a plasma-derived form of AAT (Prolastin C) was injected in mice challenged with uric acid crystals and fatty acids intra-articularly, joint swelling was strongly reduced (Figure 6C), the synovial IL-1β production was significantly inhibited (Figure 6D), and histology showed lower cellular infiltration in the joint cavity after AAT treatment (Figures 6F and 6G).

To demonstrate the clinical relevance of these findings, we thereafter assessed whether the seasonal periodicity of

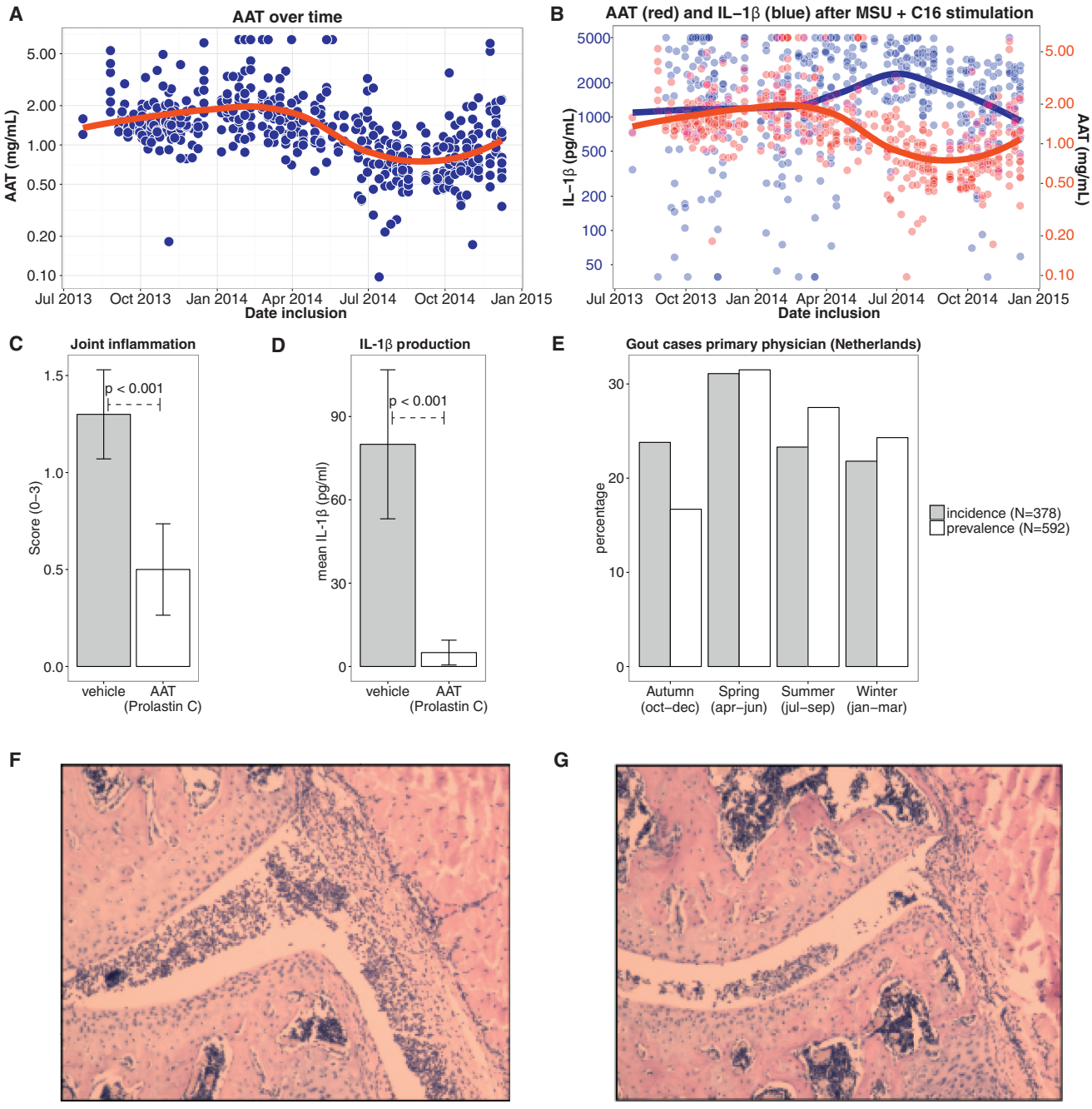


**Figure 6. AAT Effects on IL-1b Production and Gout Prevalence**

(A) Scatterplot of the seasonal response of AAT showing a decrease in summer. The line indicates the LOESS fit.

(B) Combined plots of AAT and IL-1b after influenza stimulation showing their opposite seasonal periodicity. The lines depict LOESS curves through the scatterplots.

(C) Bar plots showing decreased joint inflammation in mice injected with uric acid crystals after being injected AAT. Data are represented as mean ± SEM.

(D) Bar plots showing decreased IL-1β production in mice injected with uric acid crystals after being injected AAT. Data are represented as mean ± SEM.

(E) Number of patients presenting with gout at a primary physician in the Netherlands (n = ~800), with a clear increase in spring/summer.

(F and G) Histopathology of an inflamed knee joint of an vehicle (BSA, 100 μg/kg)-treated mouse, 4 hr after induction of gouty arthritis induced by intra-articular injection of MSU/C16.0 (300 μg/200 mM). Note the severe infiltration of cells in the joint cavity (F). Human plasma-derived AAT (Prolastin C, 100 μg/kg)-treated mouse, showing decreased inflammation (G). H&E staining, original magnification, 200×.

See also Figure S5 and Tables S5, S6, and S7.

MSU-induced cytokine responses may impact the clinical picture of gout. Indeed, retrospective assessment of the incidence and prevalence of inflammatory exacerbations in a cohort of more than 800 patients with gout identified a clear seasonality profile, with the peak in spring/summer when the cytokine production of IL-1β is highest and plasma AAT is the lowest level (Figure 6E). It is also important to note the highly negative correlation of plasma AAT with plasma IL-1β in the same plasma sample, which is consistent with AAT negatively regulating IL-1β production in vivo. All in all, this suggests that AAT is not only anti-inflammatory, but that it specifically decreases IL-1β production in a seasonal fashion and that this has a clear clinical relevance to at least one very important autoinflammatory disorder.

## DISCUSSION

The HFGP aims to understand the individual sources of variability in immune responses by studying human cytokine production capacity in response to a comprehensive panel of microbial and metabolic stimuli. We systematically investigated the factors that influence the human cytokine responses in the 500 Functional Genomics (500FG) cohort of healthy volunteers within the HFGP, after stimulation of their leukocytes with bacterial, fungal, viral, and non-microbial metabolic stimuli; while the present study assessed the impact of environmental and non-genetic host factors on cytokine responses, complementary studies investigated the impact of the genetic (Li et al., 2016) and microbiome (Schirmer et al., 2016) variability on cytokine production.

An important conclusion of the present study is that non-genetic host factors such as age or gender have a clear effect on cytokine responses, and most of these effects are cytokine- and/or stimulus-dependent. For example, old age is associated with clear defects in the production of the T-helper cytokine products IL-22 and IFNγ, while the production of monocyte-derived cytokines and IL-17 does not change with age. Changes in immune responses due to old age are well-documented, but have been performed in smaller cohorts, resulting in conflicting data: while some have reported defective TLR-induced cytokine responses in dendritic cells of elderly individuals (Panda et al., 2010), others have not identified such effects (Janssen et al., 2015). Lymphocyte defects in the elderly have been previously described (Ferrando-Martínez et al., 2011; Swain et al., 2005), and our data present an important biological correlate to this defect. The defective adaptive lymphocyte responses may thus at least partly explain the poor response to vaccination in the elderly (Weinberger et al., 2008). In contrast, the intact innate immune responses (production of monocyte-derived cytokines) can present an opportunity to initiate a new strategy of vaccination in the elderly based on trained immunity (innate immune memory) (van der Meer et al., 2015). Additionally, the presence of higher concentrations of several circulating inflammatory mediators in the elderly (such as IL-6, IL-1Ra) may be a mirror of the low-grade inflammatory condition described as "inflammaging" (Baylis et al., 2013) and that has been hypothesized to be responsible for some of the age-related chronic diseases associated with inflammation.

Another important host factor that influences immune responses is gender (Oertelt-Prigione, 2012), resulting in a differential susceptibility of men and women to infectious, autoimmune, and inflammatory diseases (Libert et al., 2010). Understanding the gender-related aspects of cytokine biology was therefore an important aim of our study. We found that monocyte-derived cytokine production was higher in men in several stimulation assays, which may contribute to the increased susceptibility of men to inflammatory diseases such as insulin resistance or atherosclerosis (Geer and Shen, 2009; Towfighi et al., 2009). In contrast, Th17 responses were higher in women using *Candida albicans* hyphae as a model stimulation system. High IL-17 production could be a driver for a higher incidence of several autoimmune diseases such as multiple sclerosis or rheumatoid arthritis in women (Gaffen, 2004; Gold and Lühder, 2008; Kotake et al., 1999; Lock et al., 2002), although this remains to be demonstrated in future studies. On the other hand, the higher *Candida*-induced IL-17 production in women may just be a mirror of more prevalent *Candida* colonization, e.g., at the level of vaginal mucosa. In contrast to age and gender, other important host-related factors such as BMI or smoking did not exert a sizeable effect on cytokine production capacity.

In addition to host factors, the immune responses of an individual are also likely to be affected by the environment, and this study aimed to comprehensively assess the role of both environmental and host factors in cytokine responses. One of the most interesting observations is the role of seasonality as a factor influencing cytokine production variability. This is biologically very relevant especially given the seasonality of many infectious diseases (Bonsall et al., 2015). A separate HFGP manuscript that assessed variation of cell counts in the 500FG cohort supports the importance of seasonality on immune parameters (Aguirre-Gamboa et al., 2016). Importantly, while previous smaller studies suggested seasonal fluctuations of vitamin D levels as the main explanation for seasonal effects (Khoo et al., 2011a, 2011b, 2012), this hypothesis is not supported by our study. The absence of direct effects on vitamin D concentrations on human cytokine responses is a crucial finding that needs further investigation: because vitamin D is seen as a possible target for health policy intervention, its true impact on biological processes in large cohorts of individuals needs to be thoroughly assessed before such policies are implemented. In contrast, our study unravels an unknown effect of seasonality on the circulating concentration of AAT, one of the most important acute phase proteins. AAT shows an inverse correlation with the production of cytokines after several stimulations, among them MSU, suggesting an anti-inflammatory effect of AAT. The biological importance of our findings is validated in an experimental model of gouty arthritis in mice (a disease caused by the formation of uric acid crystals). Even more significantly, the peak of uric acid-induced IL-1β production in summer is also validated by an increased incidence and prevalence of gout attacks during summer in a large cohort of patients. This demonstrates both the validity of the hypotheses extracted from the HFGP database, as well as the clinical impact of these processes.

All in all, the findings of this study define the main environmental and non-genetic host factors that impact immune responses. In order to comprehensively investigate the factors that influence cytokine production, complementary studies

presented in this issue of *Cell* investigate the impact of genetic variation and of the gut microbial flora on the same responses. The accompanying study by Li et al. demonstrates that genetic variability of the host has a strong effect on cytokine responses, again with the impact depending on the type of pathogen and cytokine studied (Li et al., 2016). In addition, a role of non-genetic factors for the modulation of cytokine responses is supported by the identification of important microbiome components that modulate cytokine responses, as described in the accompanying manuscript by Schirmer et al. (2016). These effects are likely mediated by microbial components or through diverse metabolites released by microbiota-diet interaction. These three complementary studies within HFGP that assess the host/environmental, genetic, and microbiome factors influencing cytokine responses provide a comprehensive picture of cytokine response variability in humans, potentially opening up new avenues for personalized medicine. Future studies and analyses of the cohorts from the HFGP will focus on the assessment of the effect of other factors (e.g., diet, metabolome), as well as on integrating these different datasets to be even more accurate in predicting and understanding the immune response against various pathogens.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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## SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and seven tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2016.10.018.

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